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Human Colorectal Cancer Cells Induce T-Cell Death Through Release of Proapoptotic Microvesicles: Role in Immune Escape

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Background & Aims: Normal and neoplastic cells release microvesicles, whose effects on the immune system still need to be elucidated. Because human colorectal cancer cells are hypothesized to escape immune recognition by expressing proapoptotic molecules, we investigated whether microvesicles bearing Fas ligand and tumor necrosis factor–related apoptosis-inducing ligand and inducing apoptosis of activated T cells are secreted by colorectal cancer cells both in vitro and in affected patients. _Methods:_ Fas ligand and tumor necrosis factor–related apoptosis-inducing ligand expression were analyzed in colorectal cancer cells and purified microvesicles by flow cytometry, Western blotting, and immunoelectron microscopy. Microvesicle tumor origin was assessed through simultaneous detection of lysosomal (CD63) and adenocarcinoma (carcinoembryonic antigen) markers. Proapoptotic activity of microvesicles was evaluated by annexin V/propidium iodide staining and caspase activation in T cells, including CD8$^+$ T lymphocytes from colorectal cancer patients. _Results:_ Colorectal cancer cells showed a granular pattern of tumor necrosis factor–related apoptosis-inducing ligand and Fas ligand expression, suggesting a secretory behavior. These proapoptotic molecules were detected on isolated microvesicles, together with class I HLA, CD63, and carcinoembryonic antigen. Microvesicles induced Fas ligand–mediated and tumor necrosis factor–related apoptosis-inducing ligand–mediated apoptosis of activated CD8$^+$ T cells generated from colorectal cancer patients. Microvesicles with comparable phenotypes and functions were found in plasma from patients with advanced disease, whereas vesicular structures expressing Fas ligand and tumor necrosis factor–related apoptosis-inducing ligand were also detected in colorectal cancer specimens. _Conclusions:_ These data show that colorectal cancer induces T-cell apoptosis through the release of Fas ligand–bearing and tumor necrosis factor–related apoptosis-inducing ligand–bearing microvesicles both in vitro and in vivo. This mechanism of immune escape has potential implications as a prognostic factor and could be targeted for the development of new antitumor therapies in colorectal cancer patients.

Intestinal epithelial cells[1] have been shown to release exosome-like microvesicles (MVs). The mechanism through which cells undergo MV secretion is not entirely known, although evidence has been provided that, at least in normal cells (eg, dendritic cells), MVs derive from the fusion of endosomal multivesicular bodies with the cell surface.[2] However, it has been postulated that the formation and secretion of MVs may differ between cells, depending on histotype and transformation.[2,3] MVs are believed to play a role in various cellular functions that mainly involve intercellular communication and immune modulation.[2,3] It is interesting to note that vesicular structures released by intestinal epithelial cells or immature dendritic cells have been shown to mediate the induction of peripheral immune tolerance to orally administered antigens or to allografts in mice through activating regulatory T cells[4] or triggering suppressive pathways by dendritic cells.[5,6]

Tumors originating from the intestinal epithelium, such as colorectal cancer (CRC), are deemed to evade immune recognition and function as immune-privileged sites through several mechanisms, including the expression of proapoptotic molecules belonging to the tumor necrosis factor (TNF) family, such as Fas ligand (FasL)

Abbreviations used in this paper: CEA, carcinoembryonic antigen; CRC, colorectal cancer; FasL, Fas ligand; IEM, immunoelectron microscopy; mAb, monoclonal antibody; MV, microvesicle; PE, phycoerythrin; TNF, tumor necrosis factor; TRAIL, tumor necrosis factor–related apoptosis-inducing ligand; TRAIL-R, TRAIL receptor.

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and possibly TNF-related apoptosis-inducing ligand (TRAIL).[7–9] FasL, a type II transmembrane protein that initiates apoptotic cascade in target cells upon binding to Fas receptor, was originally described in immune cells,[10] although its expression can be acquired by neoplastic tissues, together with the potential ability to induce apoptosis in activated Fas^{+} T cells.[11] At the same time, tumor cells should be safe from their own weapon, thanks to their resistance to Fas-induced death.[12] Other proapoptotic molecules of the TNF family, such as TRAIL, seem to be expressed in tumor cells.[13] TRAIL, which is expressed by different immune cells and plays a role in T cell–mediated and natural killer cell–mediated tumor surveillance,[14] initiates apoptosis through the engagement of its death receptors TRAIL receptor (TRAIL-R)1 and -R2 and is believed to selectively induce the death of tumor cells.[15] However, its expression in tumor cells suggests a potential involvement in tumor immune escape as well.[16]

In this article, we report for the first time that CRC cells exploit MV release for delivering FasL- and TRAIL-mediated apoptotic signals to antitumor T cells. This phenomenon, detectable in vitro in CRC cell lines as well as in the blood and tumor specimens of advanced CRC patients, may have crucial implications in the maintenance of immune tolerance to CRC.

Materials and Methods

Colorectal Cancer Lines, Colorectal Cancer Patients, and Purification of Released Microvesicles

The CRC line SW403 was purchased from the American Type Culture Collection (Manassas, VA), and 1869 col (provided by Dr C. Maccalli, Istituto Superiore di Sanità, Rome, Italy)[17] and CRC28462 were short-term lines generated from liver metastases of CRC patients. All the lines stained positively for pan-epithelial (BerEP4), cytokeratin (CD18, LP34, and MNF116), and class I HLA markers and were negative for the fibroblast marker 5B5. These lines formed colonies in soft agar, were tumorigenic in severe combined immunodeficiency disease mice, and were found to bear adenomatous polyposis coli and/or Ras mutations (P. Dalerba and G. Parmiani, unpublished data, 2004). The lines were negative for mycoplasma contamination, as routinely tested by modified nested polymerase chain reaction.[18] MVs from cell supernatants were purified by serial centrifugations.[19] MV production by CRC lines seemed to be constitutive, because equal numbers of cells release comparable amounts of vesicles when maintained under similar culture conditions (data not shown). MVs were separated from blood samples[19] (15 mL) of 10 CRC patients (Dukes stage C and D) before surgery and from healthy donors upon written informed consent and approval by the Istituto Nazionale Tumori of Milan.

Antibodies and Flow Cytometry

The following monoclonal antibodies (mAbs) were used: FasL (NOK-1); TRAIL (RIK-2); CD63 (H5C6), either pure or phycoerythrin (PE) conjugated (BD Pharmingen, San Diego, CA); carcinoembryonic antigen (CEA) (Col-1; Abcam Ltd, Cambridge, UK); class I HLA (W6.32 hybridoma); TRAIL-R1 to -R4 (Apotech Corporation, Epalinges, Switzerland); and isotype-matched immunoglobulin G (BD Pharmingen). Purified MVs were bound to latex beads (LB30; Sigma-Aldrich, Milan, Italy) and incubated either with PE-mAbs (FasL, TRAIL, and CD63) or, for indirect staining, with primary mAbs (CEA and class I HLA), followed by biotinylated anti-mouse immunoglobulin (Amersham Biosciences, Milan, Italy) and PE-streptavidin (Molecular Probes, Eugene, OR). Samples were analyzed by FACSCalibur and CellQuest software (both from Becton Dickinson).

Immunocytochemistry and Immunoelectron Microscopy

Immunocytochemistry and immunoelectron microscopy (IEM) were performed as previously described.[20] Immunocytochemistry staining was performed with alkaline phosphatase/anti–alkaline phosphatase or peroxidase/anti-peroxidase (Dako, Carpinteria, CA) methods. For IEM, ultrathin cryosections were incubated with specific mAbs and revealed with protein A gold conjugates of different sizes (5 or 10 nm, as appropriate).[20] IEM on purified MVs was performed by the immunonegative stain technique.[21] Tumor specimens were fixed, embedded, frozen, sectioned, and stained as described for cell pellets. Samples were examined with a Philips 208 transmission microscope (FEI Company, Hillsboro, OR).

Western Blotting

Cells and MV fractions were lysed and quantified by Lowry assay, and equal amounts of protein were subjected to gel electrophoresis and blotted onto a polyvinylidene difluoride membrane (Amersham). Membranes were incubated with mAbs that recognized FasL, TRAIL (HS501; Alexis, Lausanne, Switzerland), CD63, and CEA. The binding of the antibodies was detected by enhanced chemiluminescence (SuperSignal; Pierce, Rockford, IL). Staining with anti–class I HLA mAb (HC-10; provided by Dr S. Ferrone, Roswell Park Cancer Institute, Buffalo, NY) recognizing this membrane specific marker known to be enriched in MV fractions[22] was also performed to evaluate equal protein loading of cellular and MV proteins. All MV preparations were checked with appropriate mAbs for contaminating proteins derived from other cellular compartments such as the mitochondria (mAb1273 mAb; Chemicon International, Temecula, CA), endoplasmic reticulum, and Golgi apparatus (aanti-Bip/GRP78 and -GM130 mAbs, respectively; both BD Pharmingen).

Analysis of Apoptotic Activity

Activated T cells (human Jurkat cells) were cultured with MVs purified from supernatant or plasma for 12 and 48

hours, respectively; stained with annexin V/propidium iodide (Bender MedSystems, Vienna, Austria) or fluorescein isothiocyanate-VAD-fmk (CaspGLOW Fluorescein Active Caspase Staining Kit; MBL, Naka-Ku Nagoya, Japan); and analyzed by FACSCalibur and CellQuest software. For blocking experiments, cells or MVs were preincubated with anti-Fas (ZB4; Upstate Biotechnology, Lake Placid, NY), z-VAD-fmk, and TRAIL (RIK-2) mAbs. The proapoptotic activity of MVs was also tested on resting or activated (1 week of stimulation with $CEA_{571-579}$ peptide and 300 IU/mL interleukin 2)[23] peripheral blood mononuclear cells purified from CRC patients. Apoptosis was analyzed on $CD8^+$ (allophycocyanin-conjugated mAb; BD Pharmingen) T cells. Susceptibility to TRAIL and FasL was tested by using recombinant FasL (SuperFasLigand; Alexis), TRAIL (R&D Systems, Minneapolis, MN), and activating anti-Fas mAb (CH11; Upstate Biotechnology).

Modified Carcinoembryonic Antigen Radioimmunoassay

Radioimmunoassay (RIA) used for CEA measurement in CRC patients (CEA IRMA CT; Radim, Pomezia, Italy) was modified to allow the simultaneous detection of CEA and CD63 on CRC-derived MVs in supernatants and plasma samples. Latex beads were coated with CD63 mAb and incubated with purified MVs, and the formed complexes were incubated with iodine 125–labeled CEA antibody (Radim). Radioactive uptake was counted by a γ-counter (GMI, Albertville, MN).

Results

Expression of FasL and TRAIL in Human Colorectal Cancer Cells and Their Released Microvesicles

Expression of both FasL (Figure 1*A*) and TRAIL (Figure 1*B* and *C*), as detected by immunocytochemistry on CRC cell lines, displayed a granular distribution that was apparently confined to the intracellular compartments. TRAIL staining seemed to vectorially accumulate either at the cell-to-cell contact sites or at the center of pseudocrypts formed in culture (Figure 1*C*). IEM showed FasL and TRAIL localization in defined intracellular MVs (approximately 50–100 nm), primarily located in proximity to (Figure 1*D* and *E*) or fused to the cell membrane, but also detectable in the extracellular environment (Figure 1*F*), thus suggesting a secretory behavior. IEM of MV fractions purified from CRC cell supernatant showed the presence of 50–100-nm organelles that expressed both FasL (Figure 1*G*) and TRAIL (Figure 1*H*), apparently at the membrane level. The expression of both molecules was confirmed by flow cytometry (Figure 1*I*, FasL; Figure 1*J*, TRAIL). FasL and TRAIL were also detected by Western blotting as bands of approximately 42 kilodaltons (FasL; Figure 1*K*) and approximately 32 kilodaltons (TRAIL; Figure 1*L*), corresponding to the membrane-bound form of the full-length proteins.

Phenotypic Characterization of Colorectal Cancer–Released Microvesicles

When analyzed by IEM, isolated MVs expressed CD63 (Figure 2*A*), a protein of the tetraspanin family that is found on MVs originating from the lysosomal compartment,[2,3] but stained negative for markers of other intracellular compartments (Golgi apparatus; Figure 2*B*). Flow cytometry and Western blot showed the expression of CD63 (Figure 2*C* and *D*) and class I HLA molecules (Figure 1*K* and *L*, lower panels; Figure 2*C*). MVs released by SW403 and CRC28462 cells stained positive for CEA, whereas MVs derived from 1869 col cells expressing heterogenous CEA levels[17] displayed barely detectable levels of CEA protein (Figure 2*C* and *E*).

FasL- and TRAIL-Mediated Proapoptotic Activity of Colorectal Cancer–Released Microvesicles

MVs from CRC cell lines induced dose-dependent apoptosis (annexin V/propidium iodide) in activated human T cells (Figure 3*A*). Apoptosis was mediated by FasL and TRAIL, because it was efficiently blocked by anti-Fas or TRAIL mAb (Figure 3*B*), and involved caspase activation, as shown by the almost complete abrogation of MV-induced apoptotic death in T cells by pan-caspase inhibitor (Figure 3*C*). Resting lymphocytes from CRC patients were resistant to MV-, recombinant FasL–, or TRAIL-induced death (Figure 3*D*). However, after in vitro activation with the CRC antigen CEA (provided as HLA-A2–binding peptide),[23] major FasL- and TRAIL-mediated apoptosis was observed in $CD8^+$ T cells (Figure 3*E*) that was blockable by the addition of anti-Fas and TRAIL mAbs. Conversely, CRC cells did not undergo apoptosis in the presence of their corresponding MVs or recombinant FasL and TRAIL (Figure 3*F*). This resistance was not due to the absence of appropriate death receptors, because CRC cells expressed Fas and TRAIL-R2 (Figure 3*G*), but instead it might be ascribed to downstream events.

Detection of Proapoptotic Microvesicles in Plasma of Colorectal Cancer Patients and Colorectal Cancer Specimens

MVs derived from CRC patients, but not from healthy donors, expressed CEA and CD63, as shown by Western blot (Figure 4*A* and *B*). By using a modified RIA, we could simultaneously detect the expression of both CD63 and CEA molecules and, therefore, specifi-

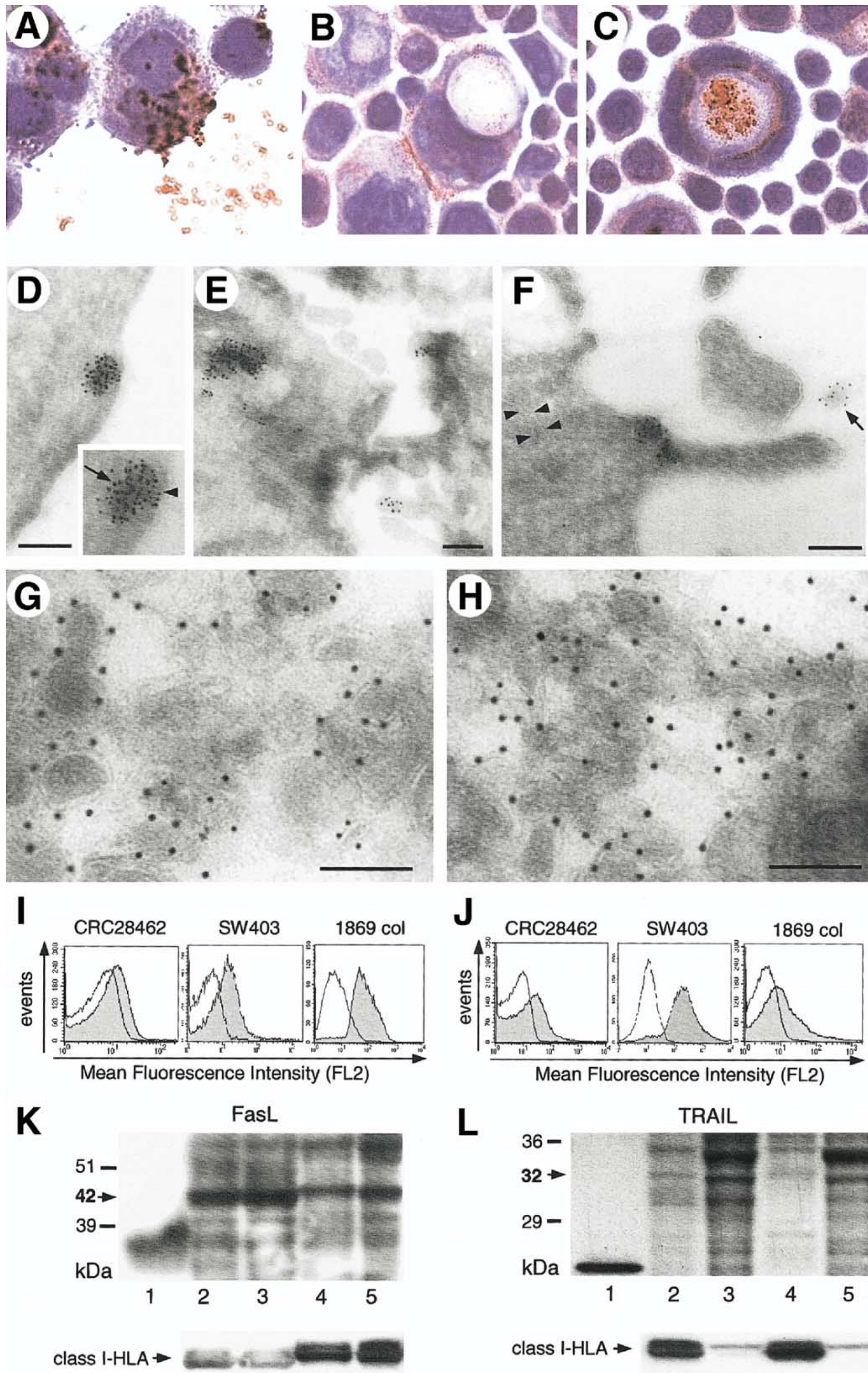

Figure 1. Expression of FasL and TRAIL in CRC cells and released MVs. (*A–C*) Immunocytochemistry of FasL (*A*) and TRAIL (*B* and *C*) in CRC cells representative of the cell lines used, showing the granular distribution pattern of both proapoptotic molecules (original magnification, 2500×). (*D–F*) IEM of FasL (large dots, 10 nm; *arrow*) and TRAIL (small dots, 5 nm; *arrowhead*) in human CRC cells shows double-positive MVs (*F*; *arrowheads*, endoplasmic reticulum; *arrow*, released MV). (*G* and *H*) IEM of FasL (*G*) and TRAIL (*H*) in MVs purified from CRC cell supernatants (*bars* = 0.1 μm). (*I* and *J*) FasL (*I*) and TRAIL (*J*) expression in CRC cell line–derived MVs detected by flow cytometry (white histograms: isotype-matched immunoglobulin G). (*K*) Western blot of FasL expression: recombinant (rec)FasL (*lane 1*) migrates at 35 kilodaltons, whereas full-length FasL in 1869 col (*lane 2*) and SW403 (*lane 3*) cell lysates and MV (1869 col, *lane 4*; SW403, *lane 5*) migrates at approximately 42 kilodaltons. *Lower panel*: Class I HLA expression (44 kilodaltons; loading control), which seemed enriched in MVs as compared with cell lysates.[22] (*L*) Western blot of TRAIL expression: recTRAIL (*lane 1*) migrates at 24 kilodaltons, whereas full-length TRAIL in lysates of 1869 col (*lane 3*) and SW403 cells (*lane 5*) and MV (1869 col, *lane 2*; SW403, *lane 4*) migrates at 32–35 kilodaltons. Additional bands correspond to the glycosylated proteins (higher bands) and breakdown pattern products (lower bands). *Lower panel*: Class I HLA expression (44 kilodaltons; loading control). kDa, kilodaltons.

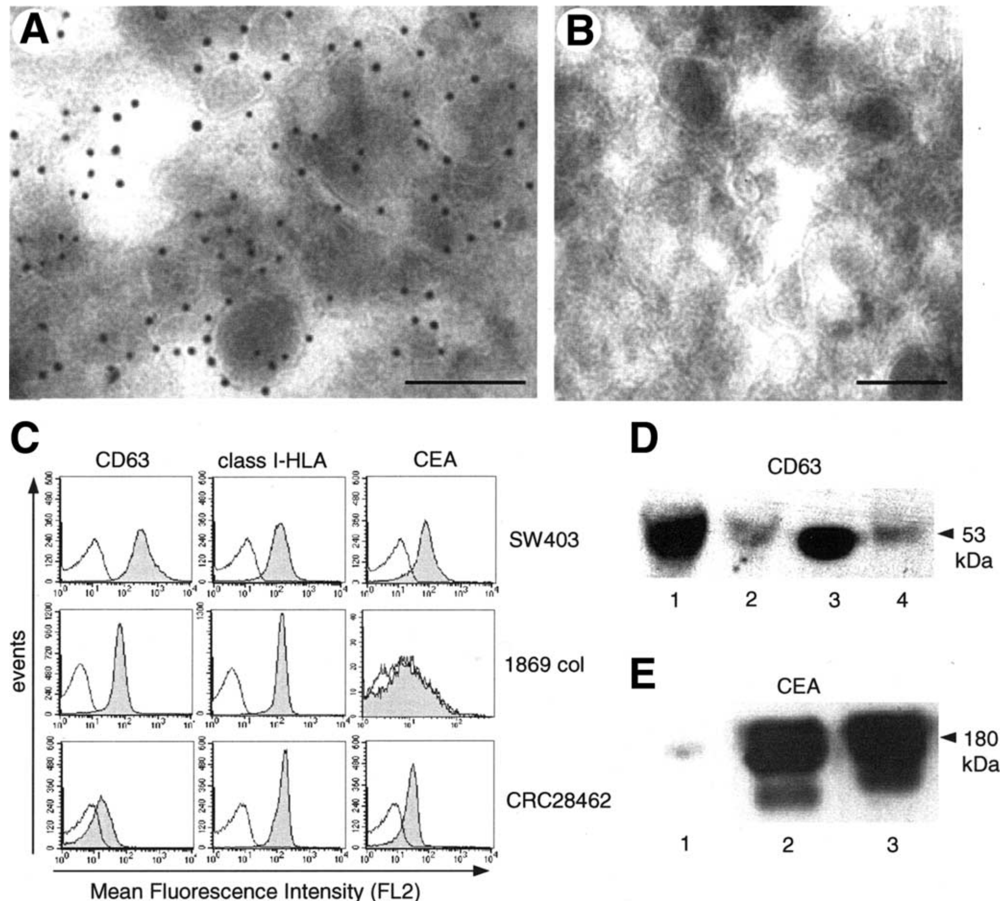

Figure 2. Expression of CD63, class I HLA, and CEA in CRC-released MVs. (*A* and *B*) IEM of MVs staining positive for CD63 (*A*) and negative for Golgi marker (*B*) (*bars* = 0.1 μm). (*C*) Flow cytometry of CD63, class I HLA, and CEA expression (white histograms: isotype-matched immunoglobulin G). (*D*) Western blot of CD63 expression in MVs from 1869 col (*lane 1*), SW403 (*lane 2*), phytohemagglutinin-activated Jurkat cells (positive control; *lane 3*), and CRC28462 (*lane 4*). (*E*) Western blot of CEA expression in MVs from 1869 col (*lane 1*), SW403 (*lane 2*), and CRC28462 (*lane 3*). kDa, kilodaltons.

cally identify MVs derived from CRC (SW403), because MVs of melanoma (501mel) cells showed no labeling (Figure 4*C*). This approach allowed us to show that MVs purified from the plasma of CRC patients were actually secreted by CRC cells in vivo (Figure 4*D*). Western blotting of MVs from the plasma of CRC patients, but not from healthy donors, showed the expression of FasL and TRAIL (approximately 42 and 32 kilodaltons, respectively), corresponding to the full-length proteins (Figure 4*E* and *F*). MVs derived from plasma of CRC patients exerted FasL- and TRAIL-mediated apoptosis on activated T cells, as compared with healthy donor–derived MVs (Figure 4*G*). The release of FasL- and TRAIL-bearing MVs by CRC cells could also be observed in vivo. Indeed, IEM performed on CRC lesions (liver metastasis) immediately processed after surgical excision showed the presence of vesicular organelles specifically expressing FasL and TRAIL at an intercellular and intracellular level (Figure 4*H*). The staining was confined to the vesicles, without any evidence of nonspecific staining on collagen fibrils. These results confirm that membrane-bound FasL and TRAIL are associated with MVs and released into the tumor microenvironment of CRC patients.

Discussion

Here we report that human CRC cells induce apoptosis of T lymphocytes through the release of FasL- and TRAIL-bearing MVs. These lysosome-derived organelles, characterized by a 50–100-nm diameter and a high content of cytosolic and membrane proteins derived from the producing cell, are released by CRC in vitro and are found in plasma and tumor specimens from patients with advanced CRC. These data shed new light on the role of FasL in immune evasion of CRC. The acquired expression of this molecule, which is considered to play a key role in CRC progression, occurs at early stages of colon carcinogenesis,[24] correlates with disease spreading,[25] and is associated with the presence of apoptotic

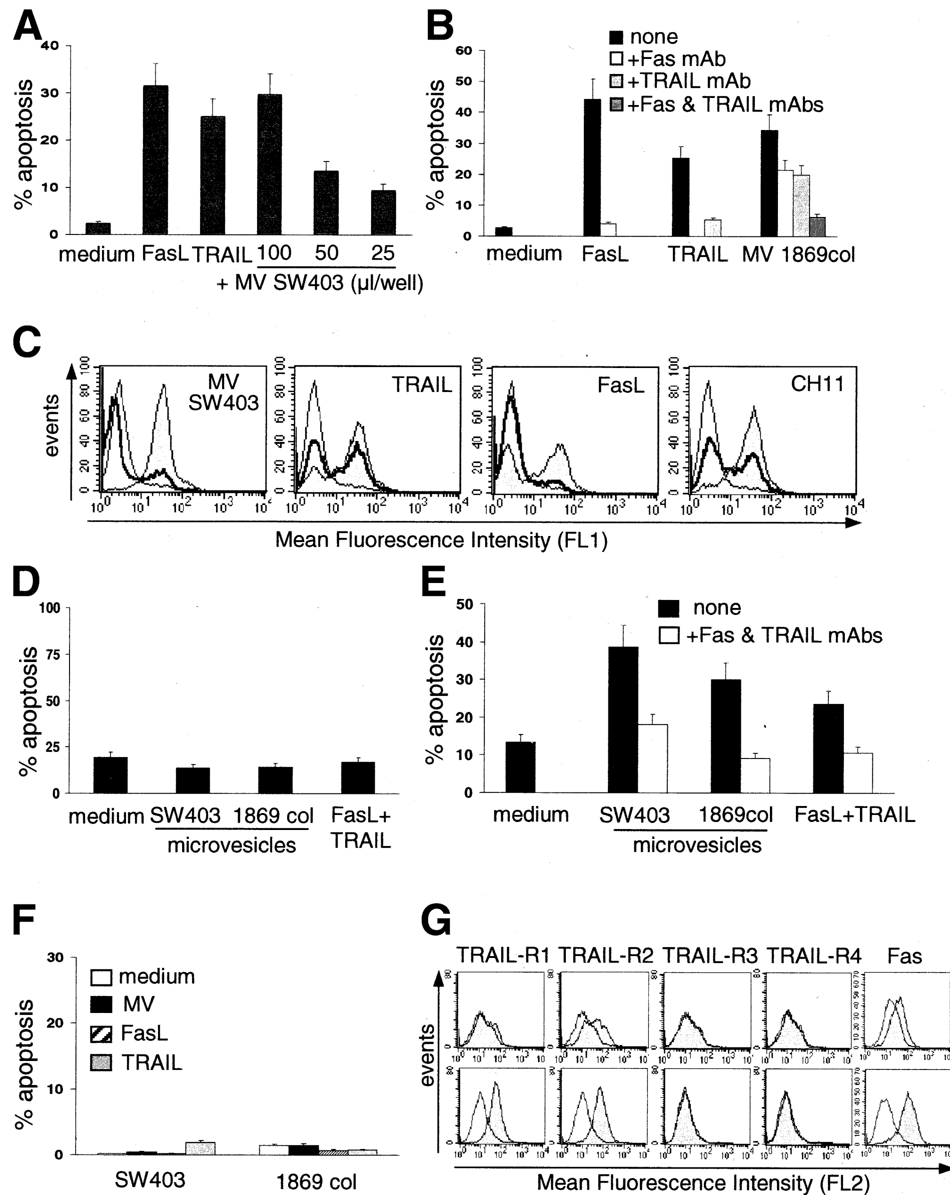

Figure 3. Proapoptotic activity of CRC cell line–derived MVs. (*A*) Dose dependency of the apoptotic activity of MVs (SW403) on activated T cells (Jurkat) in comparison with recFasL and TRAIL. (*B*) Specificity of FasL- and TRAIL-mediated apoptosis by MVs (1869 col) and recombinant proteins, showing that anti-Fas or -TRAIL mAbs decreased apoptosis (annexin V/propidium iodide staining). (*C*) Caspase activation in Jurkat cells treated with CRC-derived MVs, recFasL, and TRAIL or the agonist anti-Fas mAb CH11. *Thin line*, untreated cells; *gray area*, treated cells; *bold line,* treated cells preincubated with the pan-caspase inhibitor z-VAD-fmk. (*D*) Percentage of apoptotic cells in resting peripheral blood mononuclear cells (PBMCs) from CRC patients after incubation with CRC-derived MVs (SW403 and 1869 col) or recFasL and TRAIL. (*E*) Percentage of apoptotic cells in anti-CEA CD8$^+$ T lymphocytes (raised in vitro from the same PBMCs as in panel *D*) after incubation with CRC-derived MVs or recFasL and TRAIL. Blocking with anti-Fas and -TRAIL mAbs decreased apoptosis. (*F*) Susceptibility of CRC cells to CRC-derived MVs or recFasL and TRAIL. (*G*) Flow cytometry analysis of death receptor expression of CRC cell lines (SW403, *upper panel*; 1869 col, *lower panel*), showing the presence of Fas, TRAIL-R2, or TRAIL-R1 and the absence of the decoy receptors TRAIL-R3 and -R4.

lymphocytes at the tumor site.[26] The secretory pathway of proapoptotic MVs, which is shared by other tumor histotypes such as melanoma,[20] represents an efficient strategy for CRC cells to deliver death signals to antitumor T lymphocytes without the need for direct cell-to-cell contact.

Additionally, we show the involvement of TRAIL in the proapoptotic activity of CRC-released MVs. TRAIL expression has been shown in CRC,[13] but its potential role in these cells is still poorly understood. Although tumor cells are commonly believed to be sensitive to TRAIL-induced apoptosis,[14] we found these cells to be completely refractory to autocrine MV-induced cell death. Indeed, mechanisms that mediate TRAIL resistance through mutational inactivation of the proapoptotic Bcl-2 homologue Bax have been recently described

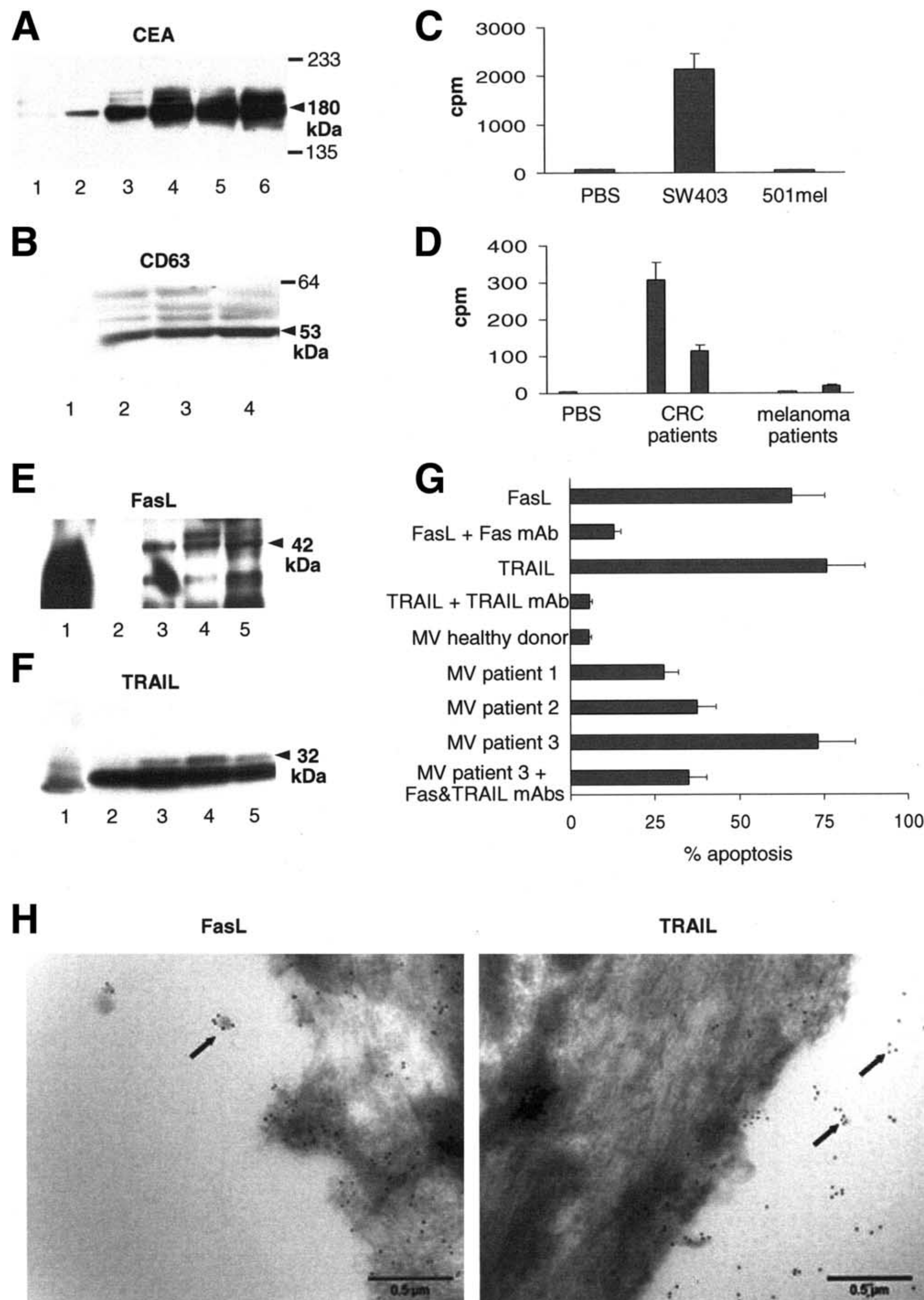

Figure 4. Detection of proapoptotic MVs in the plasma of CRC patients and tumor specimens. (*A*) Western blot of CEA expression in the lysates of MVs purified from the plasma of CRC patients (lanes 2–6) or 1 healthy donor (*lane 1*). (*B*) Western blot of CD63 expression in patient-derived MVs (*lanes 2–4*) or 1 healthy donor (*lane 1*). (*C*) CEA/CD63 modified RIA of purified MVs. CD63-coated latex bead/MV complexes (SW403 or 501mel) were analyzed for CEA expression with iodine 125–labeled CEA mAb. Only CRC-derived MVs (SW403) showed radioactive uptake. (*D*) CEA/CD63 modified RIA on MVs derived from CRC or melanoma patients. Only CRC patient–derived MVs showed radioactive uptake. (*E* and *F*) Western blots of FasL and TRAIL expression in patient-derived or healthy donor–derived MVs. (*E*) RecFasL (*lane 1*) migrates at 35 kilodaltons, whereas full-length FasL migrates at approximately 42 kilodaltons in the MVs of CRC patients (*lanes 3–5*), but not in MVs derived from 1 healthy donor (*lane 2*). (*F*) RecTRAIL (*lane 1*) migrates at 24 kilodaltons, whereas full-length TRAIL in the MVs of CRC patients migrates at approximately 32 kilodaltons (*lanes 3–5*). No full-length TRAIL was observed in MVs derived from 1 healthy donor (*lane 2*). (*G*) Patient-derived MVs induce T-cell apoptosis. Annexin V/propidium iodide staining of activated T cells (Jurkat) treated with recFasL, TRAIL, and patient-derived MVs (patients 1–3) or 1 healthy donor is shown. FasL and TRAIL mediation was shown by blocking with anti-Fas and -TRAIL mAbs. Spontaneous apoptosis of Jurkat cells (10% ± 2%) was subtracted from obtained values. (*H*) IEM staining for FasL (*left*) and TRAIL (*right*) on a CRC lesion (liver metastasis) frozen immediately after surgical resection. Note that FasL and TRAIL are selectively confined to vesicles detected intercellularly and within CRC cells (*arrows*) (*bars* = 0.5 μm). cpm, counts per minute; PBS, phosphate-buffered saline; kDa, kilodaltons.

in CRC,[27] whereas natural resistance to TRAIL has also been reported in normal colonic epithelium, despite the local expression of TRAIL and its receptors.[28]

The release of subcellular organelles (including exosomes) in physiological and pathologic conditions is presently an object of interest. These structures, produced through an active process, have been characterized by proteomic analysis and are defined by a hallmark profile that includes the presence of tetraspanin family proteins, HLA molecules, heat shock proteins, ezrin, and other proteins involved in cytoskeleton function.[2] In addition, MVs express cytosolic and membrane proteins deriving from the original cell repertoire, including tumor antigens.[19,20] CRC-released MVs described here shared most of these phenotypic features and expressed the typical CRC marker CEA, whose secretory pattern as a membrane-bound protein associated with MVs has been previously described in colonic epithelial cells.[29]

Although MV secretion could be physiological in normal intestinal epithelium,[1] this phenomenon seems to be greatly exacerbated in CRC cells, as suggested by the constitutive MV release reproducibly observed in CRC cells, which occurs through a pathway involving cytoskeleton activity (L.R., unpublished data). The expression of functional molecules, together with their small size, could then allow MVs produced at the tumor site to circulate and reach different organs. Indeed, we found that MVs with the same phenotypic and proapoptotic features as those released in vitro by CRC cells are detectable in plasma from metastatic CRC patients and can be found in CRC tumor lesions. This suggests that the secretion of proapoptotic MVs may occur in vivo as well. CRC cells may thereby locally and systemically influence the host environment through a broad array of modulating signals delivered by secreted MV. Indeed, previous studies of subcellular organelles released by intestinal epithelial cells in murine models have shown their migration to different immune sites and their possible involvement in transferring immunologic information from the intestine to the immune system.[30] Similarly, vesicles of possible tumor origin were found in malignant effusions of patients with different tumor histotypes.[19]

Because MV release is likely used by CRC cells to turn off the cellular arm of antitumor immune responses, pharmacological strategies aimed at selectively abrogating this process in vivo could become novel therapeutic tools for this disease. Furthermore, tumor-released MVs in plasma could represent a new marker of aggressiveness and prognosis in CRC patients.

References

1. Van Niel G, Raposo G, Candalh C, Boussac M, Hershberg R, Cerf-Bensussan N, Heyman M. Intestinal epithelial cells secrete exosome-like vesicles. Gastroenterology 2001;121:337–349.
2. Fevrier B, Raposo G. Exosomes: endosomal-derived vesicles shipping extracellular messages. Curr Opin Cell Biol 2004;16: 415–421.
3. Denzer K, Kleijmeer MJ, Heijnen HF, Stoorvogel W, Geuze HJ. Exosome: from internal vesicle of the multivesicular body to intercellular signaling device. J Cell Sci 2000;113:3365–3374.
4. Karlsson M, Lundin S, Dahlgren U, Kahu H, Pettersson I, Telemo E. "Tolerosomes" are produced by intestinal epithelial cells. Eur J Immunol 2001;31:2892–2900.
5. Peche H, Heslan M, Usal C, Amigorena S, Cuturi MC. Presentation of donor major histocompatibility complex antigens by bone marrow dendritic cell-derived exosomes modulates allograft rejection. Transplantation 2003;76:1503–1510.
6. Morelli AE, Larregina AT, Shufesky WJ, Sullivan ML, Stolz DB, Papworth GD, Zahorchak AF, Logar AJ, Wang Z, Watkins SC, Falo LD Jr, Thomson AW. Endocytosis, intracellular sorting, and processing of exosomes by dendritic cells. Blood 2004;104:3257–3266.
7. Marincola FM, Jaffee EM, Hicklin DJ, Ferrone S. Escape of human solid tumors from T-cell recognition: molecular mechanisms and significance. Adv Immunol 2000;74:181–273.
8. Dalerba P, Maccalli C, Casati C, Castelli C, Parmiani G. Immunology and immunotherapy of colorectal cancer. Crit Rev Oncol Hematol 2003;46:33–57.
9. O'Connell J, O'Sullivan GC, Collins JK, Shanahan F. The Fas counterattack: Fas-mediated T cell killing by colon cancer cells expressing Fas ligand. J Exp Med 1996;184:1075–1082.
10. Siegel RM, Chan FK, Chun HJ, Lenardo MJ. The multifaceted role of Fas signaling in immune cell homeostasis and autoimmunity. Nat Immunol 2000;1:469–474.
11. O'Connell J, Bennett MW, O'Sullivan GC, Collins JK, Shanahan F. The Fas counterattack: cancer as a site of immune privilege. Immunol Today 1999;20:46–52.
12. O'Connell J, Bennett MW, Nally K, Houston A, O'Sullivan GC, Shanahan F. Altered mechanisms of apoptosis in colon cancer: Fas resistance and counterattack in the tumor-immune conflict. Ann N Y Acad Sci 2000;910:178–192.
13. Inoue H, Shiraki K, Yamanaka T, Ohmori S, Sakai T, Deguchi M, Okano H, Murata K, Sugimoto K, Nakano T. Functional expression of tumor necrosis factor-related apoptosis-inducing ligand in human colonic adenocarcinoma cells. Lab Invest 2002;82: 1111–1119.
14. Takeda K, Smyth MJ, Cretney E, Hayakawa Y, Kayagaki N, Yagita H, Okumura K. Critical role for tumor necrosis factor-related apoptosis-inducing ligand in immune surveillance against tumor development. J Exp Med 2002;195:161–169.
15. Wiley SR, Schooley K, Smolak PJ, Din WS, Huang CP, Nicholl JK, Sutherland GR, Smith TD, Rauch C, Smith CA, Goodwin RG. Identification and characterization of a new member of the TNF family that induces apoptosis. Immunity 1995;3:673–682.
16. Giovarelli M, Musiani P, Garotta G, Ebner R, Di Carlo E, Kim Y, Cappello P, Rigamonti L, Bernabei P, Novelli F, Modesti A, Coletti A, Ferrie AK, Lollini PL, Ruben S, Salcedo T, Forni G. A "stealth effect": adenocarcinoma cells engineered to express TRAIL elude tumor-specific and allogeneic T cell reactions. J Immunol 1999; 163:4886–4893.
17. Maccalli C, Li YF, El-Gamil M, Rosenberg SA, Robbins PF. Identification of a colorectal tumor-associated antigen (COA-1) recognized by $CD4^+$ T lymphocytes. Cancer Res 2003;63: 6735–6743.
18. Uphoff CC, Drexler HG. Detection of Mycoplasma contaminations in cell cultures by PCR analysis. Hum Cell 1999;12:229–236.

19. Andre F, Schartz NE, Movassagh M, Flament C, Pautier P, Morice P, Pomel C, Lhomme C, Escudier B, Le Chevalier T, Tursz T, Amigorena S, Raposo G, Angevin E, Zitvogel L. Malignant effusions and immunogenic tumour-derived exosomes. Lancet 2002; 360:295–305.
20. Andreola G, Rivoltini L, Castelli C, Huber V, Perego P, Deho P, Squarcina P, Accornero P, Lozupone F, Lugini L, Stringaro A, Molinari A, Arancia G, Gentile M, Parmiani G, Fais S. Induction of lymphocyte apoptosis by tumor cell secretion of FasL-bearing microvesicles. J Exp Med 2002;195:1303–1316.
21. Tokuyasu KT. A technique for ultracryotomy of cell suspensions and tissues. J Cell Biol 1973;57:551–565.
22. Mears R, Craven RA, Hanrahan S, Totty N, Upton C, Young SL, Patel P, Selby PJ, Banks RE. Proteomic analysis of melanoma-derived exosomes by two-dimensional polyacrylamide gel electrophoresis and mass spectrometry. Proteomics 2004;4:4019–4031.
23. Nagorsen D, Keilholz U, Rivoltini L, Schmittel A, Letsch A, Asemissen AM, Berger G, Buhr HJ, Thiel E, Scheibenbogen C. Natural T-cell response against MHC class I epitopes of epithelial cell adhesion molecule, her-2/neu, and carcinoembryonic antigen in patients with colorectal cancer. Cancer Res 2000;60:4850–4854.
24. Bennett MW, O'Connell J, Houston A, Kelly J, O'Sullivan GC, Collins JK, Shanahan F. Fas ligand upregulation is an early event in colonic carcinogenesis. J Clin Pathol 2001;54:598–604.
25. Shiraki K, Tsuji N, Shioda T, Isselbacher KJ, Takahashi H. Expression of Fas ligand in liver metastases of human colonic adenocarcinomas. Proc Natl Acad Sci U S A 1997;94:6420–6425.
26. Houston A, Waldron-Lynch FD, Bennett MW, Roche D, O'Sullivan GC, Shanahan F, O'Connell J. Fas ligand expressed in colon cancer is not associated with increased apoptosis of tumor cells in vivo. Int J Cancer 2003;107:209–214.
27. LeBlanc H, Lawrence D, Varfolomeev E, Totpal K, Morlan J, Schow P, Fong S, Schwall R, Sinicropi D, Ashkenazi A. Tumor-cell resistance to death receptor-induced apoptosis through mutational inactivation of the proapoptotic Bcl-2 homolog Bax. Nat Med 2002;8:274–281.
28. Strater J, Walczak H, Pukrop T, Von Muller L, Hasel C, Kornmann M, Mertens T, Moller P. TRAIL and its receptors in the colonic epithelium: a putative role in the defense of viral infections. Gastroenterology 2002;122:659–666.
29. Baranov V, Yeung MM, Hammarstrom S. Expression of carcinoembryonic antigen and nonspecific cross-reacting 50-kDa antigen in human normal and cancerous colon mucosa: comparative ultrastructural study with monoclonal antibodies. Cancer Res 1994;54:3305–3314.
30. Van Niel G, Mallegol J, Bevilacqua C, Candalh C, Brugiere S, Tomaskovic-Crook E, Heath JK, Cerf-Bensussan N, Heyman M. Intestinal epithelial exosomes carry MHC class II/peptides able to inform the immune system in mice. Gut 2003;52:1690–1697.

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